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Review

The pleiotropic effects of heterologous Bax expression in yeast

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Abstract

The finding that the heterologous expression of Bcl-2 proteins in yeast elicits effects that resemble their roles in metazoan apoptosis has contributed to the increasing use of this organism as a model for the study of apoptotic regulation. The pro-apoptotic Bax protein, for example, localizes to the yeast mitochondria, where it acts to promote alterations in mitochondrial physiology and cell death, similar to its ascribed mode of action in higher organisms. These observations lead to the hypothesis that the heterologous Bcl-2 proteins impinge on conserved elements of the apoptotic machinery in yeast. We herein provide a retrospective of the studies aimed at both testing this general hypothesis and investigating the mechanisms of the Bcl-2 proteins using yeast, with a particular emphasis on Bax. We also discuss the evidence for pleiotropic roles of Bax in yeast apoptosis.

Keywords: Bax; Bcl-2 protein; Yeast apoptosis

1. Introduction: The Bcl-2 family members are critical regulators of apoptosis

Apoptosis is a genetically programmed form of cell death that is important for the survival of multicellular organisms by virtue of its functions in eliminating damaged or infected cells [1–4]. Further, loss of normal apoptotic responses can lead to uncontrolled cell growth, contributing to the development of tumours [5]. A number of single cell organisms, including the yeast *Saccharomyces cerevisiae*, have been shown to undergo programmed cell death (PCD) [6,7]. Given that yeast cells have been used extensively to understand the framework of a number of basic biological systems [8], it stands to reason that our understanding of PCD should therefore be greatly enhanced by the current studies of PCD in yeast. Although initially controversial, there is now very little doubt that yeast PCD is similar to the process of intrinsic apoptotic cell death that occurs in mammalian cells [9,10]. This is exemplified by the fact that the yeast genome encodes for a number of orthologous genes that have been shown to be important in mammalian apoptosis. Yeasts have an AIF (Apoptosis Inducing Factor *AIF*), metacaspase (*YCA1*), an IAP (inhibitor of Apoptosis Protein; *BIR1*), OMI/Htr2A (Nuclear Mediator of Apoptosis; *NMA111*), DJ-1 (*HSP31*) as well as a nuclease (*TAT-D*) that is a strong candidate to be involved in DNA degradation during apoptosis [11–15]. As in metazoans, overexpression of the apoptotic proteins can serve to initiate or enhance cell death in yeast, while strains lacking any of these genes show a decreased response to a number of different death stimuli.

Members of the Bcl-2 family of proteins ($n \geq 22$) are important regulators of the metazoan apoptotic process [16]. They are divided into subfamilies based on their apoptotic and anti-apoptotic nature as well as the presence or absence of different Bcl-2 Homology (BH) domains [17,18]. Bcl-2 contains all four BH domains and is a widely studied member of the Bcl-2 anti-apoptotic subfamily. Bax is the best-studied member of the pro-apoptotic Bcl-2 proteins that contain multiple BH domains. In response to pro-apoptotic stimuli, members of the Bax-like protein family act on the mitochondria to induce changes such as the release of cytochrome *c* (cyt *c*) [17–19]. Many additional BH-domain containing proteins have been studied to lesser degrees, including the pro-apoptotic Bad, Bak, and Bim, among others, as well as the anti-apoptotic Bcl-xL, BFL-1 (A1), Mcl-1, and CED-9 [17,20].

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While Bcl-2 proteins have been identified, a great deal of evidence suggests that the complete functional repertoire of Bax and Bcl-2 is not well understood [16]. Although yeast do not have genes encoding Bcl-2 proteins, the heterologous expression of mammalian Bax in yeast nevertheless induces a suppressible lethal phenotype that is associated with characteristics of metazoan apoptosis, including phosphatidylserine externalization, chromatin condensation, DNA breakage, and the formation of apoptotic bodies (Table 1) [21]. Since the initial demonstration that Bax is lethal when expressed in yeast, numerous groups have used yeast as a model system to study the function of Bax [22,23]. Studies have focused on different facets of Bax that range from determining its structure/function, to examining its role in mediating the effects on prions, to determining how it interacts with the mitochondria to using the lethal effects of Bax to screen for novel anti-apoptotic genes. Here we will give a historical perspective of Bax in yeast and we review the establishment of this model system as a powerful platform for the study of the structural/functional/mechanistic properties of Bax and other Bcl-2 family members. We also present the current knowledge on the mechanisms by which Bax promotes deleterious effects in yeast and discuss the use of classical yeast-based approaches, such as functional suppressor screens, to gain functional insights into the evolutionary conserved process of apoptosis.

2. The establishment of yeast as a tool for functional studies on Bax

2.1. Bax expression in yeast induces a programmed cell death with apoptotic features

The initial observation that Bax expression confers a lethal phenotype in yeast was made during the course of yeast two-hybrid studies aimed at examining Bax/Bcl-2 interactions [24]. Expression of Bax fused to either DNA-binding or *trans*-activation domains in yeast cells, under the control of a strong promoter, resulted in marked loss of clonogenic capacity that was confirmed to be due to cell death. Further, the effect was specifically suppressed by co-expression of anti-apoptotic Bcl-2, Bcl-xL, and Mcl-1, indicating that both pro- and anti-apoptotic Bcl-2 family members retained certain aspects of their function when heterologously expressed in yeast [24]. Subsequent studies confirmed a lethal phenotype upon expression of Bax, unfused and lacking a signal peptide, under the control of the *GAL1/GAL10* promoter [25]. These observations lead to the hypothesis that the mammalian Bcl-2 proteins act on elements of a conserved endogenous yeast machinery to mediate effects on viability [26]. Importantly, Bax-mediated cell death in yeast, as in mammalian cells, involves a regulated insertion into mitochondrial membranes and mitochondrial dysfunction leading to the release of cyt *c* and apoptosis, supporting the hypothesis that Bax exerts effects in yeast that are comparable to those in its native setting [23].

A seminal study in the field of yeast apoptosis involved the identification of typical markers of mammalian apoptosis in cells mutated for CDC48 [27]. These findings strongly suggested that the basic framework of apoptosis is conserved in lower eukaryotes. The first description of apoptotic phenotypes in yeast induced by members of Bcl-2 family involved the expression of pro-apoptotic human Bak in *Schizosaccharomyces pombe* [28]. The specific phenotypes reported included nuclear envelope breakdown and chromatin condensation and fragmentation, which are characteristics of metazoan apoptosis. The deleterious effects of Bak were dependent on the BH3 domain and suppressed by co-expression of Bcl-xL and Bcl-2, but not by a mutant form of Bcl-2 that does not prevent apoptosis in mammalian cells [29]. Shortly thereafter, similar

Table 1
Bcl-2 and Bcl-2-related proteins and their reported effects in yeast

	Phenotypes observed on isolated yeast mitochondria or upon heterologous expression in yeast.	References
Pro-apoptotic Bcl-2 proteins		
Bax	Loss of mitochondrial membrane potential, and cytochrome *c* release. Induction of oxidative stress, growth-inhibition, and cell death. Elicits cell death upon expression in *Schizosaccharomyces pombe*.	[20,28,27,33,37,106]
Bad	Sensitizes cells to the effects of Bax, yet was also reported to enhance the pro-survival activity of Bcl-xL, Bcl-2 and A1.	[44,167]
Bak	Loss of mitochondrial membrane potential. Induction of cell death upon expression in *Schizosaccharomyces pombe.*	[26,27,37]
Bcl-xS	Enhances the pro-survival activity of Bcl-xL, Bcl-2, and A1.	[37,167]
Bim	Loss of mitochondrial membrane potential, cytochrome *c* release and cell death. Enhances the death-inducing activity of Bax.	[122,166]
BimS	Sensitizes cells to the lethal effects of Bax.	[166]
BNip3	No effect observed.	[44]
BNip3L	No effect observed.	[44]
Noxa	No effect observed.	[44]
Puma	Sensitizes cells to the effects of Bax.	[44]
tBid	Disrupts mitochondrial state-3 respiration and ATP production. Sensitizes cells to Bax-induced cytochrome *c* release and cell death.	[43,44]
Anti-apoptotic Bcl-2 proteins		
A1	Protects from Bax- and Bak-induced cytotoxicity.	[37]
Bcl-2	Promotes cell survival. Increases resistance to oxidative stresses.	[33,37,51]
Bcl-xL	Prevents Bax-induced growth-inhibition and cell death. Increases resistance to oxidative stresses.	[27,37, 106]
BFL-1	Protects from Bax-induced cell death.	[168]
CED-9	Promotes resistance to oxidative stresses.	[51]
Mcl-1	Protects from Bax- and Bak-induced cytotoxicity.	[37,165]

apoptotic events induced by Bax expression in *Saccharomyces cerevisiae* were reported [30]. This report documented the phenotypes of chromatin condensation, DNA breakage, plasma membrane blebbing, as well as Annexin V-positive Bax-expressing cells, an indicator of the commonly observed metazoan apoptotic phenomenon of phosphatidylserine externalization [30]. While there has not been detection of DNA laddering, a phenotype typically observed in apoptotic mammalian cells, this has been proposed to be due to the marked distinctions in heterochromatin structure and organization [31]. Nevertheless, the findings by Ligr and others clearly indicate the appearance of hallmarks of apoptosis upon Bax expression in yeast [21,30].

2.2. The lack of apparent Bcl-2 family member orthologs in yeast has facilitated their functional analysis

The evidence described above suggesting that Bcl-2 family members impinge on conserved molecular components prompted a number of groups to study the function of these metazoan regulators of apoptosis in yeast. Importantly, yeast lack apparent orthologs of metazoan Bcl-2 family members [32]. Heterologous expression of these proteins in yeast therefore allows the study of their mechanisms of action in the absence of the network of Bcl-2 apoptotic regulators present in metazoans. This fact has facilitated the study of individual Bcl-2 proteins, since the presence of alternate endogenous forms of Bcl-2 family members with conflicting or redundant functions often makes interpretation of a given experiment problematic [16]. For instance, the demonstration that pro- and anti-apoptotic Bcl-2 proteins are able to interact leads to the hypothesis that heterodimerization represents a major regulatory mechanism between these proteins [24,33]. However, a mutant form of Bcl-xL which is unable to bind Bax retains the ability to prevent the deleterious effects of Bax in yeast [34]. Therefore, the finding that individual Bcl-2 family members retain their pro- or anti-apoptotic functions when expressed in yeast strongly suggested heterodimerization-independent functions of these proteins [34,35]. These findings are consistent with descriptions of autonomous functions for Bcl-2 proteins in both yeast and mammalian cells, such as pro-survival effects due to an intrinsic anti-oxidant function [36].

In light of their initial observations that Bax is lethal to yeast, Reed et al. used this system to study the Bcl-2 family members, including the structural determinants of Bax function [37]. The interpretation of structure/function studies performed in yeast-based systems has resolved many questions concerning the regulation of metazoan apoptosis by the Bcl-2 family [23,38]. In addition to providing the first evidence for heterodimerization-independent mechanisms by which both Bcl-2 and Bcl-xL prevent Bax-induced cell death [35,39], the functional analysis of deletion mutants of the Bcl-2 protein lead to the initial descriptions of the BH domains [35]. Mutants deleted for the transmembrane domain (TMD) of Bcl-xL, but not of Bcl-2 were unable to prevent Bax-induced cell death in yeast, suggesting divergent mechanisms of action for the two proteins [39]. Also, homodimerization-defective mutants of Bax were shown to be ineffective in eliciting a lethal phenotype, suggesting a crucial role for homodimerization in the effects of Bax [31]. Importantly, these findings were consistently validated by studies in mammalian cells [34,40].

The Bcl-2-null genetic background was further exploited in yeast engineered to allow the independent regulation of expression levels of both pro- and anti-apoptotic Bcl-2 proteins [41]. The level of a HA–Bcl-xL fusion (expressed under the control of the inducible *tetO* promoter) sufficient to protect from the effects of HA–Bax (expressed under the control of *GAL1* promoter and maintained at constant levels) remained constant over a wide range (30–40-fold difference) of HA–Bax expression levels, suggesting that heterodimerization of Bcl-xL with Bax is not required for the protective effects. Strongly supporting this conclusion is the unaltered ability of a heterodimerization-defective mutant form of Bcl-xL to prevent the effects of Bax [41]. Further, increasing the expression of HA–Bcl-xL to levels that were effective in preventing the lethal effects of HA–Bax maintained at constant levels resulted in a concomitant cytoplasmic accumulation of Bax. A quantitative analysis indicated, however, that the amount of Bax that remained inserted at the mitochondria was sufficient to induce lethality, indicating a role for Bcl-xL in preventing effects of membrane-integrated Bax [41].

Additional examples of lessons learned from the yeast-based study of Bcl-2 proteins include the demonstration of a stimulatory effect of a caspase-generated form of Bid (tBid) on cell death and cyt *c* release induced by activated Bax [42,43]. However, this stimulatory effect of tBid was not observed when co-expressed with the ground state Bax, demonstrating that Bax activation (by a tBid-independent mechanism) precludes the stimulatory action of tBid [44]. In a subsequent study, experiments using recombinant tBid incubated with isolated yeast mitochondria confirmed findings that tBid disrupts mitochondrial bioenergetics independent of other Bcl-2 family members [45].

Finally, Guscetti et al. carried out an expansive analysis of pro-apoptotic BH3-only proteins in yeast, and established three categories of such proteins based on their functional characteristics: directly killing (tBid), those that sensitize Bax/Bcl-2 expressing cells for death (Bad or puma), and BH3-only proteins that display no toxicity (BNip3, BNip3L, and Noxa) [46]. In a separate study, the BH3-only protein BimS, previously hypothesized to promote apoptosis by inhibiting anti-apoptotic Bcl-2 proteins, was demonstrated to enhance the lethal effects of Bax in yeast, thereby arguing against a dependency for other Bcl-2 proteins in BimS function [47]. These findings illustrate the usefulness of yeast in studying the function of Bcl-2 family members by virtue of its ‘clean’ genetic background.

2.3. Yeast as a model to study Bcl-2 proteins and their relationship to other heterologous apoptotic regulators

2.3.1. Studies on the apoptotic regulatory molecules of Caenorhabditis elegans in yeast

Seminal genetic studies by Horvitz et al. in *Caenorhabditis elegans* resulted in the identification of important apoptotic

regulatory molecules, including CED-3, CED-4, and CED-9 [48]. CED-3 encodes for a caspase, while CED-4 and CED-9 represent orthologs of Apaf-1 and Bcl-2, respectively [49]. Following suite from the inducible yeast expression systems developed for the study of the Bcl-2 proteins [37,39], several groups investigated the properties of the *Caenorhabditis elegans* apoptotic proteins in yeast [50–53]. Early studies in *Schizosaccharomyces pombe* indicated the presence of endogenous machinery responsive to the CED proteins [53]. The heterologous expression of *ced-4* resulted in the apoptotic phenotypes of chromatin condensation and lethality, and these effects were suppressed upon co-expression of *ced-9*. The heterologous expression of either *ced-3* or *ced-4* was subsequently shown to result in lethality of *Saccharomyces cerevisiae*, in a manner suppressible by *ced-9* expression [50]. The killing activity of CED-4 was dependent on its nucleotide triphosphate-binding loop in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, as it is in *Caenorhabditis elegans*, indicating a specific mode of action for this protein in yeast [50,53]. Further, these studies provided the evidence for a caspase/CED-3-independent and CED-9-suppressible cell death pathway induced by CED-4. Refuting this notion, a subsequent report describing a reconstituted CED pathway in yeast indicated that CED-4 alone is insufficient to induce lethality, but rather that it possessed a stimulatory effect on CED-3-mediated cell death that was suppressed by CED-9 [52]. Further, the pro-survival effect of CED-9 was not observed upon induction of cell death by *ced-3* expression alone. It should also be noted that, similar to other anti-apoptotic Bcl-2 proteins, CED-9 has been demonstrated to protect yeast from the lethal effects of oxidative stress, suggesting an anti-apoptotic role independent of the CED protein network [54].

The yeast expression system was also employed to define the interplay between Bcl-2 and the *Caenorhabditis elegans* apoptotic machinery. Bcl-2 displays no inhibitory action on CED-4-mediated lethality in yeast, arguing against the previously proposed direct pro-survival role for Bcl-2 observed in *Caenorhabditis elegans* and in mammalian cells ectopically expressing *ced-3* and *ced-4* [51]. Bcl-2 expression is, however, effective in binding to and displacing the pro-apoptotic *Caenorhabditis elegans* protein EGL-1, suggesting an alternate mechanism for the observed anti-apoptotic effects of heterologously expressed Bcl-2. Based on these collective findings, yeast is clearly useful to reconstitute heterologous apoptotic regulatory networks and continued work in this system will help delineate the complete functions of the CED proteins in apoptosis.

2.3.2. Prions and Bax in yeast

More recently, Bax-induced cell death in yeast has been exploited to perform genetic and functional analyses of mammalian prion protein PrP [55,56]. Prior reports indicating that the expression of the non-infectious PrPC form of the prion protein suppresses the effects of Bax in cultured neurons [57] prompted the examination of its effects on Bax-mediated cell death in yeast. The expression of prion protein in yeast, which is handled by the secretory pathway to produce a GPI-anchored cell surface protein, rescued Bax-induced lethality [55,56]. Removal of the signal peptide and C-terminal membrane-anchoring sequences from PrP prevented its suppressive capability, suggesting that cytosolic PrP cannot prevent Bax-induced cell death. However, a subsequent report demonstrated Bax suppression by an expressing native, human PrP, which is largely excluded from the secretory pathway because of the weak nature of the heterologous signal peptide. Consistent with observations in mammalian systems [58], these findings suggest that cytosolic prion protein can suppress the effects of Bax. Therefore, yeast is an amenable model system for the study of mammalian proteins that functionally interact with Bax and can lead to biological insights into the conserved Bax-mediated mechanisms.

2.3.3. The use of Bax-expressing yeast cells to identify and characterize novel anti-apoptotic proteins

The demonstration that Bax displays lethal functions in yeast raised the prospect that suppressors of this phenotype that represent putative anti-apoptotic factors could be identified by functional screening strategies. Reed and co-workers initially used this screening strategy and reported the isolation of Bax inhibitor-1 (BI-1) from a human HepG2 hepatocarcinoma cDNA library [59,60]. BI-1 is now a well-studied protein that possesses anti-apoptotic activity across kingdoms [61]. In the years succeeding this finding, a number of heterologous anti-apoptotic proteins have been identifies by such strategies [62–74]. This use of forward genetics provides not only information regarding mechanisms of Bax-mediated metazoan apoptosis through the identification of conserved metazoan anti-apoptotic factors, but also offers mechanistic insight into the evolutionarily conserved apoptotic and anti-apoptotic programme regulated by Bax [26]. Similarly, classical genetic approaches have been used to address the molecular requirements for Bax-mediated cell death and on-going studies are likely to further advance the knowledge on apoptotic mechanisms in yeast [75]. Below, we discuss some of the heterologous Bax suppressors identified in phenotypic screens.

Bax is known to elevate ROS levels, and cellular responses to such oxidative stresses include a robust attempt to counter these elevations through a variety of mechanisms [76]. For instance, cells commonly increase the expression of anti-oxidant proteins that served to protect against increases in ROS [76,77]. Examples of such proteins identified in Bax suppressor screens include a plant Glutathione *S*-transferase/peroxidase, a soybean ascorbate peroxidase, a tomato phospholipid hydroperoxide, and a glutathione peroxidase [65,69,78]. Further, a VAMP protein isolated as another Bax suppressor was demonstrated to function downstream of the Bax-induced oxidative burst [68]. Collectively, these findings underscore the central role of ROS in cellular responses to Bax (see also Section 3.4) [21].

A human HepG2 cDNA library screen resulted in the identification of Bifunctional Apoptosis Regulator (BAR), a 450 amino acid protein containing a TMD, and RING finger and Sterile Alpha Motif (SAM) domains [74]. Initial characterization of deletion mutants of BAR allowed the prediction that the mammalian anti-apoptotic effects are independent of the RING

domain, but abrogated in the absence of the TM domain, suggesting a role for membrane localization in the function of BAR [74]. It was further shown that the SAM domain binds anti-apoptotic Bcl-2 proteins, but not Bax, and is required for BAR-mediated anti-apoptosis. Curiously, suppression of Bax-induced lethality in yeast was also dependent on the SAM domain of BAR. Given the absence of Bcl-2 family members in yeast, this suggests a conserved SAM-dependent mechanism of anti-apoptosis in yeast and warrants further investigation.

A subunit of the Ku protein complex with functions in non-homologous DNA double-strand break repair was identified as a suppressor of Bax in yeast and was found to prevent the translocation of Bax to the mitochondria, thereby preventing Bax-mediated apoptosis in mammalian cells [71]. This report indicated an anti-apoptotic function for Ku70 protein in addition to, and independent of, its role in DNA repair.

In screening for Bax suppressors, we identified the mouse sphingomyelin synthase I (SMS1α) [73]. SMS1α is an ER-localized protein that uses ceramide and phosphatidylcholine to produce sphingomyelin and diacylglycerol (DAG). Although the gene encoding SMS1 has only recently been cloned [79,80], the enzyme has long been considered to be anti-apoptotic due to its utilization of the pro-apoptotic sphingolipid ceramide [81,82]. Ceramide levels are known to increase in response to stress, analogously to ROS, and can trigger a variety of intracellular signaling responses, including apoptosis induced by prolonged elevations in ceramide levels [82]. The heterologous expression of SMS1α in yeast also conferred resistance to the deleterious effects of H_2O_2, heat, and exogenously added C2-ceramide and PHS [73]. The latter is a precursor used in the synthesis of phytoceramide (fungal ceramide). These results suggest that in addition to inducing an increase in ROS, Bax expression in yeast also leads to the accumulation of phytoceramide. Overexpression of SMS1 has been recently reported to be anti-apoptotic in mammalian cells [83].

Our screen of a human heart cDNA library has led to the identification of 72 different Bax suppressors. While a number of the proteins identified, such as Hsp72, Hsp90β, and Suv3 were previously identified as being anti-apoptotic, the majority of the clones have not. For instance, an alternatively spliced protein encoded by the human VPS24 gene, Vps24β has been characterized as a strong anti-apoptotic sequence in yeast [67]. Interestingly, the previously characterized Vps24α is not anti-apoptotic and functionally compliment the lack of yeast Vps24, whereas Vps24β does not. Also, the Bax-suppressive function of Vps24β is independent of a functional ESCRT pathway, of which the yeast Vps24 is an essential component. These findings suggest that the human VPS24 gene encodes for two functionally distinct proteins: Vps24α that functions in the ESCRT pathway, and Vps24β that suppresses the effects of Bax in yeast through an as of yet unknown mechanism.

2.4. Effects of Bcl-2 family members in other yeasts

Deleterious effects of Bax expression have been reported in other unicellular ascomycetes, including *Candida albicans*, *Kluyveromyces lactis*, and *schizosaccharomyces pombe* [84,85]. Extending the aforementioned studies involving Bak expression in *schizosaccharomyces pombe* are descriptions that this apoptotic phenotype is associated with defects in cell cycle progression and an accumulation of large vesicular structures and that Bak-induced lethality is mediated partly through an interaction with calnexin, a component of the ER quality control apparatus [86]. Unexpectedly, expression of Bax in *schizosaccharomyces pombe* results in a slow growth phenotype, not cell death. Also, while the effects of both Bax and Bak are suppressible by co-expression of Bcl-2 and Bcl-xL, co-expression of the anti-apoptotic adenoviral E1B 19K protein suppresses only the Bak-induced phenotype, further suggesting distinct mechanisms for Bax and Bak function. Of note, the *schizosaccharomyces pombe* genome encodes for the BH3-only protein SpRad9 that promotes a Bcl-2 suppressible apoptosis when expressed in mammalian cells [87]. Interestingly, SpRad9 is a critical component of a complex of proteins (the '9–1–1 complex', comprised of Rad9p, Rad1p Hus1p) that mediate DNA damage/replication checkpoint signaling [88]. While a role for SpRad9 in yeast apoptosis has not been demonstrated, the human Rad9 serves as a direct link between cell cycle checkpoint control and apoptosis, suggesting a worthwhile avenue of investigation in *schizosaccharomyces pombe* [89].

Bax expression also promotes cell death and the accumulation of endogenous ROS in the yeast *K. lactis* [85]. Interestingly, while the co-expression of Bcl-xL prevents the cytotoxic defects of Bax, the levels of ROS were not diminished. This indicates that elevated ROS in *K. lactis* does not necessarily result in cell death and that the anti-oxidant functions are distinct from the anti-apoptotic properties of Bcl-xL.

Expressing a synthetic, codon-optimized Bax-encoding sequence in the pathogenic yeast *Candida albicans* results in growth-inhibition and lethality [84]. Interestingly, fusion of Bax with eGFP resulted in a potentiated cell death response to Bax, by virtue of a hampered proteolytic degradation of the fusion protein. Visualization of this fusion protein indicated a mitochondrial localization of Bax. Further, Bax expression in *Candida albicans* causes the translocation of mitochondria from a spatially dispersed organization to a cluster in the perinuclear region. While this mitochondrial clustering phenotype has not been observed in other yeasts, there are reports of such effects in mammalian cells induced by pro-apoptotic stimuli, such as TNF-α, trail, and ceramide [84].

Recently, it was demonstrated that Bax expression in the hemibiotrophic plant pathogen *Colletrichum gloeosporioides* induces cell death with apoptotic hallmarks, and that expressing Bcl-2 prolonged lifespan and stress resistance [90]. In addition, morphogenetic changes affecting the adaptive responses to environmental factors, such as mycelium growth, conidia production and germination, as well as plant virulence, are modified by the expression of Bcl-2 and Bax. These findings suggest that a regulatory cross-talk exists between the endogenous apoptotic machinery responsive to the Bcl-2 proteins and the molecules that mediate various developmental stages in the *Colletrichum gloeosporioides* life cycle.

3. Mechanisms of Bax-induced apoptosis occurring at mitochondria

A central role for the mitochondria in Bax-mediated apoptosis has been clearly demonstrated in both metazoan and yeast cells. Activated Bax localizes to the mitochondria and promotes alterations in the mitochondrial membrane potential and permeabilization of the outer mitochondrial membrane (OMM) allowing the release of apoptogenic factors [91]. The extent to which these are conserved in yeast is as of yet uncertain. Nevertheless, there exist endogenous orthologs of metazoan genes with similar roles in the apoptotic cascade. All the available evidence suggests that heterologously expressed Bax protein impinges on evolutionarily conserved elements to induce specific effects, and is therefore thought to be a useful tool to study the regulation of the apoptotic response [23]. The demonstrated ability of Bcl-2 family members to act in yeast as they do in a native setting suggests that yeast possesses factors that perform these functions (i.e. proteins that form pores in the OMM). Interestingly, while sharing only modest similarities in sequence, certain regulators of mitochondrial fission/fusion events with roles in yeast apoptosis display pore-forming properties [92]. Further investigation should reveal whether these or other yeast proteins represent functional orthologs of Bcl-2 family members. Nevertheless, the study of Bax in yeast has provided much insight into the role of Bax at the mitochondria [23]. We present a review of these finding in the following sub-sections.

3.1. Mitochondrial targeting and attachment of Bax in yeast

Structure/function studies focused on the relationship between the determinants of mitochondrial targeting of Bax and its ability to confer phenotypes such as cyt *c* release or cell death. An N-terminal region of Bax, containing the first alpha helix (Ha1), serves as a signal required to address the protein to both yeast and mammalian mitochondria [93]. The deletion of the first 19 residues, comprising the Apoptosis-Regulating Targeting (ART) sequence of Bax enhanced the binding of Bax to the yeast mitochondria, suggesting a role for this sequence in cytosolic retention of the protein, as had been proposed by prior studies in mammalian cells [94]. This is consistent with findings that the naturally occurring Bax ψ, which lacks the ART sequence, displayed constitutive mitochondrial localization [93].

Immunofluorescence studies demonstrated that the mitochondrial localization of heterologously expressed Bax is dependent on its C-terminal TMD [31]. The TMD was also required for the cytotoxic effect observed, suggesting that mitochondrial targeting of Bax is critical for its pro-apoptotic function. The behavior of the wild type Bax protein was reconstituted in a mutant lacking the TMD by replacing the missing domain with a TMD sequence from Mas70p, a yeast mitochondrial outer membrane protein [31]. Subsequent studies contrasted these findings, however, by indicating that a His6-tagged Bax protein lacking the C-terminal TM domain displayed no effect on cyt *c* release from permeabilized yeast spheroplasts and isolated mitochondria when compared to full length Bax [95]. Also, an independent analysis demonstrated that a mutant form of Bax lacking the C-terminal domain retained its mitochondrial localization in yeast, as well as the ability to confer a lethal phenotype [96]. Interestingly, while the substitution of the C-terminal domain of Bax with the homologous sequence from Bcl-xL did not impair the mitochondrial localization, it abrogated the deleterious effects of Bax. This indicates that mitochondrial insertion is not sufficient for the lethal effects of Bax. Supporting these findings, subsequent mutagenesis of the C-terminal TM domain indicated that it is not required for the insertion of Bax into yeast mitochondria [97]. This study instead revealed that specific mutations within this domain conferred a more pronounced mitochondrial insertion and cyt *c* release phenotype, suggesting an inhibitory role for the domain with respect to these properties, similar to the N-terminal ART sequence described above.

Cardiolipin is a mitochondria-specific phospholipid that is required for the cooperative pore-forming functions of Bid and Bax [98]. Analyses using cardiolipin-deficient strains of yeast due to mutations in the cardiolipin synthase-encoding gene, *CRD1*, have led to contrasting findings with regard to the role of this lipid in Bax-mediated cell death. While one study indicated that strains lacking *CRD1* exhibited no difference in mitochondrial insertion of Bax as compared to wild type, a subsequent study suggested that strain differences may account for these discrepancies [99].

Studies in yeast were also carried out to determine the molecular targets of Bax at the mitochondria. The human Tom22p, a component of the protein import complex translocase of the outer membrane (TOM), was demonstrated to act as a mitochondrial Bax receptor [100]. Independent evidence for this came from diploid yeast strains heterozygous for TOM22 that displayed reduced levels of Tom22p present at mitochondria. There was also a significantly reduced level of Bax present in isolated mitochondria from heterozygotes, when compared to wild type mitochondria, supporting the model that Tom22p anchors Bax at the mitochondrial surface [100].

An investigation into the mechanisms of tcBid/Bax targeting to the mitochondria revealed a critical role for components of the TOM complex [43]. In contrast to the work involving Tom22p discussed above [100], the domains of the OMM exposed to the cytosol, including the cytosolic regions of Tom22p, appear to be dispensible for the tcBid/Bax-induced cyt *c* release. Also, as reported previously with regard to the effects of Bax expression [99], the extent of cyt *c* release induced by tcBid/Bax was unaltered by the deletion of the *CRD1* gene, indicating the cardiolipin-independent nature of the event. Of further interest, tcBid/Bax failed to induce cyt *c* release from mitochondria isolated from yeast expressing the temperature-sensitive allele of the pore-forming TOM complex subunit Tom40p in non-permissive conditions.

Finally, a recent study addressed the hypothesis that phosphorylation events of key serine residues mediate the conformational changes required for relocalization of cytosolic Bax to mitochondria. Substitutions of serine residues within consensus target sites for GSK3β, PKA, and Akt/PKB revealed the potential importance of the phosphorylation of these residues in Bax activation upon induction of apoptosis [101].

3.2. Bax expression promotes the release of cytochrome c

A central event in Bax-mediated apoptosis is the release of specific mitochondrial proteins from the intermembrane space into the cytosol upon permeabilization of the outer mitochondrial membrane (OMM). A number of resident mitochondrial proteins are detected in the cytosol of apoptotic cells, including cyt *c*, Smac/Diablo, Omi/Htr2A, AIF, and EndoG [102]. These released proteins have specific roles in the execution of the apoptotic programme. Omi/Htr2A, for instance, possesses a serine protease activity that has been demonstrated to cleave members of the Inhibitor of Apoptosis Protein (AIP) family, including XIAP, and thereby promote apoptosis [102]. Cyt *c* is the most extensively studied of these proteins. Upon its release, cyt *c* forms the aptly named apoptosome complex in the cytosol with caspase-9 and Apaf-1. This is essential for the activation of caspase-3, the 'executioner' caspase that cleaves a number of intracellular substrates [103].

Manon et al. first described the release of cyt *c* in yeast when studying the mitochondrial effects of Bax expression in whole cells, isolated mitochondria, and permeabilized spheroplasts [104]. As in mammalian cells, the release of cyt *c* can also be induced by other apoptotic stimuli. In yeast, these stimuli include acetic acid, human α-synuclein expression, and specific apoptosis-inducing mutations in *CDC48* [105–107], suggesting that the release of cyt *c* is also central to the yeast apoptotic programme.

While there are data suggesting a direct role of cyt *c* release in the apoptotic response [105–108], Bax retains its ability to kill yeast lacking cyt *c* [109], indicating the presence of alternative pathways through which Bax-induced cell death occurs. Also, Bax-induced cell death has been reported to occur with undetectable levels of cytosolic cyt *c* [110], and while a cytochrome *c*–GFP fusion is not released by Bax-expression under both fermentative and respiratory conditions, the endpoint of death is still observed. Therefore, cyt *c* release is not essential for Bax-mediated apoptosis in yeast [111]. Together, these results suggest that Bax-induced cyt *c* release may be an important apoptotic event, but that alternate pathways exist. This notion is consistent with reports in mammalian cells indicating the induction of apoptotic events independent of cyt *c* release [112,113].

3.3. Mechanisms regulating the release of mitochondrial factors in response to Bax expression

The release of mitochondrial factors is a crucial event during apoptosis, and therefore the mechanisms regulating OMM permeabilization are of continued interest to investigators. Despite the persistence of a number of hypotheses that explain OMM permeabilization, the steps leading to this event are largely unresolved. Because the IMM and OMM are left largely intact [23], Bax-induced OMM permeabilization is therefore thought not to be simply the result of destruction of the membranes due to excess mitochondrial swelling, as has been proposed by some groups [23]. Bax expression is associated with distinct alterations in mitochondrial structure and physiology, including a hyperpolarization of the IMM, matrix swelling, an ultimate dissipation of the IMM potential, a disruption of mitochondrial bioenergetics, and finally release of the aforementioned apoptogenic factors [102]. However, conclusive evidence on the sequence or temporal profile of these events, as well as causal associations between them has remained elusive. A distinct advantage of studying these phenomena in yeast is that in this organism, mitochondrial function can be disrupted while retaining cell viability, in contrast to higher eukaryotes, thus facilitating genetic analyses on Bax-mediated mitochondrial alterations. In the following sub-sections we review what is known regarding the Bax-induced release of yeast mitochondrial factors and discuss the studies in which the current models mediating this release have been tested in yeast. The reader is referred to additional recent reviews [23,102,114] for detailed descriptions of the current models explaining Bax-mediated OMM permeabilization in mammalian and yeast cells.

3.3.1. Bax expression induces mitochondrial changes in yeast by acting on components of the permeability transition pore complex

Early events associated with apoptosis include an increase in the permeability of the IMM to solutes <1.5 kDa in size, the mitochondrial permeability transition (MPT), and changes of the mitochondrial membrane potential ($\Delta\Psi_m$), prompting the uncoupling of the respiratory chain and release of apoptogenic mitochondrial factors [114]. A number of findings suggest a critical role for the permeability transition pore complex (PTPC) in Bax-induced alterations of $\Delta\Psi_m$ [115,116]. The PTPC is a large protein complex that spans both the IMM and OMM, and is comprised of the voltage dependent anion channel (VDAC), an ADP/ATP carrier (or adenine dinucleotide transporter; ANT) and cyclophilin D, as well as certain accessory factors [117]. While the precise role of the PTPC in Bax-induced apoptosis has not been resolved, studies in whole yeast cells and isolated mitochondria have indicated that the PTPC is required for Bax function [23].

It should be noted, as described in the sections that follow, that the role of specific PTPC components in Bax-mediated apoptosis remains a matter of controversy, with conflicting conclusions from different groups [23,109,118]. These distinct effects of Bax expression have been proposed to be due to differences in expression levels, different epitope tags fused to the native Bax protein, and metabolic differences caused by growth on different carbon sources, as well as different genetic backgrounds of the various strains used in the studies [41,109,110,119,120]. For instance, Priault et al. demonstrate a markedly reduced level of cyt *c* release induced by expressing Bax under the control of the tetracycline-responsive tetO promoter, as compared to the much stronger *GAL10* promoter [120]. Also, numerous reports describe the distinct effects of Bax when yeast cells are cultured in fermentative, semi-fermentative, or solely respiratory growth-dependent conditions [25,95,119]. Therefore, a careful examination of the growth conditions is important when comparing studies on the events associated with the expression of Bax in yeast and their genetic determinants.

Bax-induced $\Delta\Psi_m$ dissipation, as well as release of apoptogenic factors from isolated mitochondria, is prevented upon pharmacological inhibition of the PTPC in mammalian cells [121]. Further, Bax interacts specifically with the ANT subunit of the PTPC in the two-hybrid system and the two proteins co-immunoprecipitate at endogenously expressed levels in HT-29 cells. There appears also to be a requirement for a functional interaction between Bax and the ANT, since Bax-deficient cells are unresponsive to a PTCP-activating ANT ligand [110]. In support of these observations, yeast cells lacking the three ANT-encoding genes were markedly resistant to the negative effect on clonogenicity induced by LexA-fused Bax expressed under the control of the *GAL10* promoter [115]. This suggests that Bax acts to kill yeast in a conserved ANT-dependent mechanism.

Contrasting to these suggestions that the effects of Bax are mediated through ANT, the overexpression of ANT-1 induces apoptosis in mammalian cells, but does not affect the growth of yeast cells [122]. In addition, yeast lacking ANT display levels of Bax-induced apoptotic phenotypes, including cyt *c* release, profiles of $\Delta\Psi_m$, dissipation, and the MPT hallmark of mitochondrial swelling, that were unaltered when compared to wild type cells [123]. Also, ANT-deficient cells exhibit wild type kinetics of loss of clonogenicity upon induction of Bax [124]. Lastly, it should be noted that an examination of the effects of Bax on protein import into the mitochondria revealed that cells co-expressing Bax and ANT-GFP displayed decreased mitochondrial fluorescence when compared to cells expressing either ANT-GFP alone or Bax co-expressed with a mitochondrial protein (Mtp1p) that is imported though a distinct mechanism [124]. These findings lead the authors to posit that Bax may act to prevent, specifically, the import of mitochondrial carrier proteins.

Concerning the model of PTPC-mediated release of apoptogenic factors, there are data that suggest that, rather than ANT, alternate subunits of the complex are important in the process [76,77]. The aforementioned study in which the presence of ANT had no effect on mitochondrial effects of Bax had been preceded by an elegant demonstration of a direct action of Bax on VDAC in synthetic liposome preparations [125]. The authors followed these observations up by showing a significantly reduced ability of Bax to induce mitochondrial dysfunction and cyt *c* release in whole yeast cells and isolated organelles deficient in VDAC [123]. Similarly, an investigation of the mechanisms of apoptosis induced by the pro-apoptotic Bcl-2 member Bim included studies on isolated yeast mitochondria from VDAC1-deficient cells, wherein Bim was found to promote cyt *c* release and loss of $\Delta\Psi_m$ in a manner dependent on VDAC1 [126].

These results are in contrast to the findings of Priault et al. who demonstrated Bax-mediated cyt *c* release in the absence of VDAC [109,120]. Interestingly, a strain lacking the VDAC-encoding *POR1* gene displayed more pronounced Bax-induced effects, suggesting that VDAC may in fact interfere with the toxic function of Bax [118]. Also, a comparative analysis of expression levels indicated that the minimum amount of Bax protein detected in cells undergoing Bax-mediated cell death is nearly 200-fold less than the amount of VDAC present in the OMM [41]. A recent report that VDAC1, and additionally VDAC2, are not essential for the effects of Bax in yeast was published recently by Szklarz et al. while employing a functional genomics approach to identify genes that are necessary for the Bax-mediated release of mitochondrial proteins [127]. Very recently, using mitochondria isolated from VDAC-deficient cells, an additional study included the finding that VDAC is dispensible for the tcBid/Bax-induced release of cyt *c* [43]. These findings have been corroborated by strong evidence indicating that mammalian cells lacking VDAC display profiles of stress-induced MPT, cyt *c* release, caspase cleavage, and cell death that are indistinguishable, and in some cases exacerbated when compared to wild type cells [117].

3.3.2. Bax-mediated OMM pore formation independent of endogenous mitochondrial proteins

A number of observations are suggestive of an independent pore-forming role for Bax leading to the release of intermembrane space proteins. The structure of some of the Bcl-2 family members display similarity to pore-forming bacterial toxins and are able to form ion channels in synthetic membranes [128,129]. In particular, oligomeric Bax can insert into membranes and allow the release of cyt *c* [130]. The mitochondrial apoptosis-induced channel (MAC) is a high conductance ion channel, distinct from VDAC, that is induced by apoptotic stimuli [131]. MAC was found to share similarities with Bax-formed channels, and its formation is inhibited by Bcl-2 expression. Interestingly, MAC channel activity was detected in mitochondria from Bax-expressing yeast cells, confirming a correlation between the presence of Bax at the mitochondria and MAC channel activity [131]. Therefore, Bax promotes the formation of an ion channel in both mammalian and yeast mitochondria that is capable of releasing cyt *c*.

Further evidence for self-sufficient pore-forming action of Bax emerged from the recent examination of the role of protein import machineries, including Translocase of the Outer Membrane (TOM) and Sorting and Assembly Machinery (SAM) complexes, in the Bax-mediated release of mitochondrial factors [127]. The import of nascent mitochondrial proteins into isolated yeast mitochondria was unaffected by the induction of mitochondrial protein release by Bax. Further, mitochondria either treated with protease or isolated from cells lacking different components of the mitochondrial fission, fusion, or pre-protein import machinery display a robust release of mitochondrial proteins in response to Bax expression. These results clearly show that the Bax-induced release of yeast intermembrane space proteins occurs while the protein import into the mitochondrial matrix ensues and suggests that it is independent of endogenous mitochondrial proteins [127]. Surprisingly, the pore diameter analyses in this study allowed the release of cytochrome *b1* complex, a 230 kDa multisubunit structure roughly 10-fold larger than the capacity of the Bax-induced MAC-like channel described above [131]. These data are also inconsistent with the previously described finding that a GFP-tagged cyt *c*, a fusion protein that is less than 230 kDa in size, is not released into the cytosol upon Bax expression [111]. Thus, while there are data to suggest a pore-forming role for

Bax in the induction of apoptogenic factor release, the biochemical and biophysical properties of the pore are likely modulated by membrane constituents, potentially explaining the differences observed by groups using custom membrane preparations [123].

3.4. The role of oxidative stress in Bax-mediated yeast apoptosis

Reactive oxygen species (ROS) are natural products of aerobic metabolism that mediate an array of biological effects, including inflammatory and immune responses, proliferation, cellular senescence, and by virtue of their damaging effects on all classes of cellular material, necrosis [76]. In addition, oxidative stress, caused by elevations in intracellular ROS levels, is a central mediator of apoptosis in most, if not all, cell types examined, including yeast [77]. The evidence for a role for oxidative stress in yeast apoptosis includes the analysis of apoptotic responses in cells lacking or overexpressing genes encoding proteins preventing oxidative stress and observations that exogenous anti-oxidants protect from apoptosis [54]. Further, the detection of elevations in intracellular ROS in yeast has been observed in response to a variety of apoptotic stimuli, such as acetic acid and Bax expression [21,107].

Madeo et al. demonstrated that elevations in intracellular ROS were necessary and sufficient to induce apoptosis in yeast providing a strong argument for an evolutionarily conserved consequence of stress-induced oxidative stress [21]. In addition, the authors demonstrated that the accumulation of ROS induced by Bax expression was inhibited by co-expression of Bcl-xL. Free radical spin traps were also effective in preventing the Bax-induced apoptotic events, including chromatin condensation, DNA strand breakage, and cell death. Bax-induced ROS accumulation is not observed in a [rho^0] strain and is dependent on genes encoding specific components of the respiratory chain, including VDAC and cyt *c* [110]. These results are in agreement with prior studies [119,120], which demonstrated that the lethal effects of Bax were markedly less severe when yeasts are grown under anaerobic conditions. Additionally, a number of subsequent findings strongly suggested that Bax-induced apoptosis is mediated through elevations in ROS leading to oxidative stress and have been substantiated by subsequent reports [64,67,69,132].

In addition to oxidative stress caused by the accumulation of ROS, alternate oxidative pathways exist that have been suggested to mediate apoptotic responses in response to stressful stimuli [133]. The involvement of alternate oxidative processes in apoptosis has also been documented in yeast expressing Bax. For instance, Bax expression markedly reduced both the total amount of mitochondrial fatty acids and the ratio of unsaturated/saturated fatty acids, and that these changes were due to mitochondrial lipid peroxidation [119]. The incorporation of exogenous fatty acids promoted Bax-induced lethality and treatment with inhibitors of lipid peroxidation prevented Bax-induced cell death, suggesting that lipid peroxidation is a direct mediator of Bax-induced lethality. Despite chemical scavenging of Bax-induced ROS, the Bax-induced effect on lipid peroxidation persisted, suggesting that lipid peroxidation is not a consequence of Bax-induced ROS generation, but rather one of lipid-oxidizing enzyme activation.

The mechanism by which Bax promotes ROS accumulation remains unknown. Attempting to address this, Reekmans et al. reasoned that H_2O_2 treatment likely generates ROS directly, bypassing the mechanisms by which Bax-induced ROS formation occurs [134]. Therefore, transcripts differentially regulated by Bax expression, but not by an equally toxic dose of H_2O_2, represent candidate proteins acting to mediate ROS formation induced specifically by Bax. Using microarray- and RT-PCR-based strategies, the authors confirmed a Bax-specific transcriptional response for 4 genes (*MLS1*, *OYE3*, *ICY2*, and *BTN2*). Following the rationale outlined above, strains lacking these genes were predicted to be defective in the pro-oxidant and lethal effects of Bax. Indeed, the *OYE3* gene, encoding an NADPH dehydrogenase for an unknown substrate, was required for Bax-induced cell death. Further, while Bax-induced membrane lipid peroxidation was impaired in the Δ*oye3* strain, H_2O_2-induced cell death and lipid peroxidation responses were exacerbated. These results further supported a role for lipid peroxidation in the effects of Bax, and suggested that *OYE3* regulates Bax-induced accumulation of ROS.

The critical role of ROS accumulation and oxidative stress inreceived in revised form Bax-mediated yeast apoptosis discussed above has been substantiated by subsequent findings from other groups [64,66,69]. Cells possess a variety of mechanisms that protect from oxidative stress, including the enzymatic modulation of ROS directly (i.e. by catalases, superoxide dismutases, or peroxidases), as well as the regulation of the redox state of the intracellular milieu through anti-oxidants (i.e. glutathione, thioredoxin) [38,77]. Further support for the hypothesis that oxidative stress is critical for Bax-induced yeast apoptosis is provided by studies on the isolation of suppressors of Bax-induced cell death that function directly in the oxidative stress defense systems, including the soybean APX, and the tomato GST/GPX and PHGPx (see also Section 2.3.3) [65,69,78]. In addition to providing insight into Bax function, the GST/GPX prevented Bax-induced reductions in intracellular glutathione levels, suggesting that glutathione depletion is a mechanism by which Bax promotes lethality [78]. It should be noted also that 4 of the 6 Bax-suppressive clones identified in the report describing the GST/GPX were homologues of known anti-oxidant proteins [78]. Finally, the tomato QM protein acts to promote accumulation of antioxidative proline, thereby preventing oxidative stress [135]. This protein confers protection from Bax-induced ROS accumulation and reductions in viability, supporting the hypothesis that ROS mediate the lethal effects of Bax. These findings collectively underscore the importance of oxidative stress defense systems in the response to Bax.

3.5. Oxidative phosphorylation in Bax-mediated yeast apoptosis

It is clear that Bax-mediated apoptosis involves the action of the protein at mammalian mitochondria. Early investigations

indicated a requirement for the oxidoreductive environment of the yeast mitochondria for Bax-induced lethality, but not for growth-inhibition [25]. While wild type cells displayed a loss in viability in response to the induction of Bax, cells lacking a mitochondrial genome ([rho^0], or 'petites') were completely resistant to these effects. Subsequent works toward identifying the genetic determinants of Bax-mediated cell death have repeatedly indicated an important role for the oxidative phosphorylation machinery and respiratory status in Bax-mediated yeast apoptosis [75,118,136]. These studies further exemplify the amenability of yeast to functional genomics approaches, and provide additional examples as to the advantages of using a facultative aerobe in the study of the mitochondrial mechanisms of Bax-mediated apoptosis, since interfering with oxidative phosphorylation in mammalian cells results in lethality. For instance, *ATP4*, a nuclear gene encoding a critical subunit of the F_1–F_0 proton pump (F_1–F_0–ATPase), was isolated in a screen for yeast mutants resistant to Bax-mediated lethality [75]. The examination of haploid and heterozygous diploids lacking *ATP4* revealed a requirement of the gene for Bax-induced lethality. Pharmacological inhibition of the F_1–F_0–ATPase also abrogated the lethal effects of Bax, confirming the requirement of a functional Atp4p-containing proton pump. Finally, while Bax-induced death was completely abrogated in cells mutated for an additional component of the F_1–F_0–ATPase (*ATPδ*), the kinetics of death were shown to be only markedly delayed (not completely abrogated) in [rho^0] cells. The absolute requirement of F_1–F_0–ATPase function (of which partial activity is maintained in [rho^0] cells), but not that of the mitochondrial genome, suggests a specific requirement for the proton pump, and not a general dependency on respiratory competence. Supporting such a conclusion, studies in mammalian cells demonstrated a protection from Bax-dependent apoptosis by sublethal doses of an F_1–F_0–ATPase inhibitor, but not by inhibition of an alternate complex of the respiratory chain [75].

Direct evidence for a pH-regulated, *ATP4*-dependent mechanism in the effects of Bax in yeast was obtained by using a pH-sensitive mutant of GFP (pH-GFP) that responds to changes in pH between 7.0 and 8.5 by nearly linear increases in fluorescence emissions [136]. Changes in emission profiles of a mitochondrial matrix targeted pH-GFP indicated a Bax-induced alkalinization of yeast mitochondria in wild type cells that was not observed in cells lacking *ATP4*. In addition, there was a concomitant *ATP4*-dependent cytosolic acidification upon expression of Bax. The authors of these studies posit that changes in the IMM potential or of the pH in the intermembrane space due to the deficiency of the proton pump interfered with the insertion of Bax into the membrane or Bax channel activity.

In addition to confirming the importance of *ATP4*, further evidence indicating a role for oxidative phosphorylation is provided from the analysis of Bax-mediated lethality in a panel of strains deficient in specific mitochondrial protein-encoding genes [118]. Strains lacking genes essential for growth on non-fermentative carbon sources (i.e. *COX4*, *ATP4*, *PET9*, *CYC3*, *COX7*, *QCR7*, as well as [rho^0]) displayed significantly increased resistance to the deleterious effects of Bax, whereas strains that retained respiratory competence (i.e. those lacking *SDH3* and *NDI1*) responded similar to wild type. An additional independent study indicated that cells lacking *ATP4* and *ATP2* displayed a dramatic resistance to Bax-induced cell death [110]. Further, a permanent respiratory defect due to inhibition of oxidative phosphorylation is a direct consequence of Bax induction [118,124]. These are compelling arguments supporting the notion that Bax in yeast, as mammalian cells, functions to promote specific alterations in mitochondrial physiology and function [118].

Given the central role of the mitochondria in apoptosis, one might expect the respiratory status of the cell to modulate the response to Bax expression. Priault et al. demonstrated that the extent to which Bax expression induces cell death is directly related to the respiratory ability of yeast [120]. This is reconciled with findings that high cytosolic ATP/ADP ratios (present in respiring cells) promote the cytotoxic effects of Bax [104]. However, cells grown in fermentative conditions are also killed upon expression of Bax, albeit with a significantly delayed response. These data argue for a cell death-promoting function for Bax independent of oxidative phosphorylation. This notion is consistent with an examination by Kissova et al. that revealed that both ANT-deficient and [rho^0] strains displayed wild type rates of Bax-induced loss of clonogenicity [124].

In addition to cyt *c*, Manon et al. reported a decrease in the amount of cytochrome *aa3*, and therefore a decrease in cyt *c* oxidase complex in response to Bax expression [104]. Subsequent reports indicated a decrease in the level of cyt *c* oxidase present at the IMM as a consequence of Bax expression [137]. This decrease was later found to be the result of degradation of a cox subunit, cox2p, through the action of a Bax-activated protease termed Yme1p. Interestingly, Bax-mediated cell death is delayed in cells lacking *YME1*, suggesting that Bax-mediated apoptosis occurs through decreasing the levels of Cox2p. Cell death is not abrogated, however, indicating alternate pathways of cell death execution and possibly reflecting a preference for distinct pathways used for apoptosis depending on respiratory status.

4. Bax expression in yeast elicits pleiotropic effects

Upon expression in yeast, Bax localizes to mitochondria and alters the physiology of this organelle in a manner comparable to the effects of Bax in metazoan systems [23]. A specific role for Bax at the mitochondria leading to apoptosis has been supported by a number of observations using whole cells, as well as purified mitochondria. These include many of the observations discussed above, such as the protective ability of anti-apoptotic Bcl-2 family members, and both microscopic and biochemical demonstrations that Bax undergoes regulated localization to mitochondria [23]. While Bax expression in yeast is clearly sufficient to induce yeast apoptosis, alternative hypotheses have emerged that question the specificity with regard to the function of Bax in yeast. For instance, heterologous expression of Bcl-2 can alleviate the oxidant hypersensitivity of yeast cells lacking the gene encoding

superoxide dismutase 2 (Δ*sod2*), and therefore serves a protective function independent of Bax [36]. Thus, while the Bax-suppressive functions of anti-apoptotic Bcl-2 proteins may indeed reflect specific modes of killing, it is difficult to determine the contribution of their innate anti-oxidant activity in these effects. Additionally, in contrast to reports in mammalian cells describing the activation of Bax by stress-induced ROS generation [138], the production of endogenous ROS in yeast appears to be a downstream effect of Bax function [10], analogous to what is observed in yeast challenged with exogenous stressful stimulus.

A number of groups have reported alternative phenotypes associated with Bax expression in yeast, such as Bax-induced alterations in vacuolar and protein transport pathways, autophagy, and mitophagy [139–141]. Certain suppressor screens have indicated that Bax displays pleiotropic effects. For instance, an evolutionarily conserved suppressor of the effects of Bax in yeast (BI-1) has functions at the ER [142,143], suggesting a mitochondria-independent pathway of Bax-mediated apoptosis. It should be noted that apoptosis in mammalian cells has been reported as a consequence of ER-localized Bax and Bak proteins [144]. Further, a screen for yeast mutant displaying resistance to the lethal effects of Bax expression identified *UTH1*, a gene that was also shown to be required for rapamycin-induced autophagy [145]. These observations suggest that Bax expression in yeast may induce a stress response not exclusive to the mitochondria and thereby mediate pleiotropic effects. Below we review further evidence for the pleiotropic effects of Bax expression in yeast.

4.1. The effects of Bax are independent of canonical pro-apoptotic regulators

The identification of a caspase-like protein encoded by the *YCA1* gene represents a seminal discovery in the study of yeast apoptosis, providing support for the notion that molecular pathways that are conserved between higher eukaryotes mediate the apoptotic programme [12]. *YCA1* is one of a growing number of yeast genes, including *AIF1*, *NMA111*, and *NUC1*, with apoptotic roles in response to different apoptotic stimuli, such as H_2O_2, acetic acid, heat stress, osmolarity, and aging [11–13,146]. Early analyses indicated that Bax-mediated lethality in yeast is unaffected by the co-expression of the cowpox virus-encoded CrmA, a known inhibitor of caspase activity [39]. Consistently, it was subsequently reported that Bax-induced cell death occurs independent of *YCA1* (*MCA1*) [46] and that the lack of *YCA1* had no effect on the loss of plating efficiency induced by Bax expression [147]. Furthermore, cells deficient in the pro-apoptotic *AIF1* gene unexpectedly exhibited more pronounced losses of plating efficiency as compared to wild type cells [147]. In addition, no detectable increase in caspase activity is detected in response to Bax induction. Therefore, the effects of Bax on cell death in yeast appear to be independent of the canonical apoptotic mediators. It should be noted, however, that caspase-independent apoptosis in response to a variety of stimuli, including Bax expression, has also been reported in mammalian systems [148,149].

4.2. Bax expression promotes plasma membrane integrity

Bax expression in yeast prevents the ethanol-induced permeabilization of the plasma membrane observed during induction of apoptosis under fermentative conditions [150]. This extra-mitochondrial effect of Bax parallels the maintenance of mammalian plasma membrane integrity that distinguishes apoptotic from necrotic cell death. Recently, the maintenance of plasma membrane integrity by Bax was found to be independent of both *YCA1* and *AIF1*, suggesting that the effect is not mediated through the apoptotic programme [147].

4.3. Bax overexpression induces autophagic cell death and mitophagy

Autophagy is a genetically programmed process whereby portions of the cytoplasm are enveloped by double-bilayer vesicles (autophagosomes) and delivered to the vacuole. This leads to the ultimate degradation of the autophagosomal contents and is thought to serve the purpose of nutrient recycling during periods of starvation [151]. Accumulating evidence suggests a role for autophagy in the regulated degradation of whole organelles to promote adaptation to different growth conditions [151]. For instance, the selective removal of mitochondria occurs through a specialized form of autophagy, termed mitophagy [152]. By promoting adaptation in times of stress, therefore, autophagy is thought to promote cell viability. A seemingly contradictory notion has emerged of late through the analysis of morphologic and genetic data suggesting an active role for autophagy in programmed cell death. One model stemming from work in both yeast and mammalian cells posits that while a low-level of autophagy is indeed protective, an increased level may serve to promote death [153]. However in the absence of clear molecular signals that would regulate such a transition, autophagy is not considered a *bona fide* programmed cell death process, but is denoted as "programmed cell death with autophagy" [154]. Some reports also describe a caspase-independent form of programmed cell death associated with extensive autophagocytosis as Type II cell death, whereas the traditional characteristics of apoptosis define Type I cell death [155].

Initial visualizations of yeast cells expressing Bax using electron microscopy (EM) indicated the presence of autophagic, rather than apoptotic, phenotypes, such as vacuolarization [30]. Further, the genetic determinants of autophagy have been shown to co-regulate apoptotic responses in both yeast and mammalian cells in some conditions. For instance, the induction of the human ortholog of *APG5*, a gene that is necessary for autophagosome formation in yeast and mammalian cells, is induced by apoptotic stimuli [156]. Also, the anti-apoptotic Bcl-2 protein displays anti-autophagic effects in both yeast and mammalian cells [153]. Autophagic and apoptotic programmed cell death responses are indeed distinct, however, because the former can lead to a loss of cell viability in the absence of hallmarks associated with the latter, and vice-versa [154]. It has been proposed that it is the extent to which the autophagic programme is induced that determines the ultimate effect on cell viability [153].

4.3.1. Uth1p: A link between the apoptotic and autophagic-inducing effects of Bax?

Identified in a screen for mutants displaying resistance to Bax-induced cell death, the *UTH1* gene encodes for an OMM-localized protein that represents a potential molecular determinant of apoptotic or autophagic effects of Bax [145]. In strains deleted for *UTH1*, Bax is targeted to the mitochondria and retains the ability to promote cyt *c* release. However, additional phenotypes that are crucial for Bax-induced apoptosis, including effects on lipid peroxidation, ROS production, and plasma membrane stability, displayed a dependency on the presence of *UTH1*. Also, expression of *UTH1* under the control of the *GAL1* promoter was sufficient to promote cell death. These data indicate a critical role for *UTH1* in the programmed cell death response to Bax expression in yeast.

Observations that Δ*uth1* cells are resistant to the deleterious effects of other stressful stimuli such as H_2O_2, carbon and nitrogen starvation, and elevated temperatures [157], prompted the examination of the role of Uth1p in programmed cell death with autophagy. Interestingly, cells lacking *UTH1* are resistant to the *APG5*-dependent decrease in viability induced by rapamycin, indicating that Uth1p may function to prevent autophagy [145]. Further, this phenotype is observed only when cells are grown on respiratory carbon sources, indicating the requirement for mitochondria in the protection against autophagy-mediated cell death.

A recent report indicated primarily autophagic, rather than apoptotic, phenotypes concomitant with loss of plating efficiency induced by Bax [147]. These phenotypes included increased accumulation of Atg8p, activation of a vacuolar alkaline phosphatase, and the detection of autophagosomes and autophagic bodies by EM. Bcl-2 or Bcl-xL co-expression prevented the autophagic characteristics of Bax expression. Strains deleted for genes essential to the process of autophagy (*ATG4*, *ATG5*, *ATG7*, *ATG8*, *ATG15*, *ATG16*), however, displayed either similar or more pronounced rates of clonogenicity. Therefore, while Bax induces autophagy, the autophagic process is not necessary for the Bax-induced loss of plating efficiency.

A previous study suggested that *UTH1* mediates the process of selective mitochondrial autophagy, but does not exert effects on cytosolic autophagy [152]. Given the role of *UTH1* in Bax-induced programmed cell death, a role for the process of mitophagy in the effects of Bax was examined [147]. Visualization of a fluorescent mitochondrial probe revealed a more pronounced Bax-induced fragmentation of the mitochondrial network in a Δ*uth1* strain as compared to wild type cells. Furthermore mitophagy-dependent degradation of the mitochondria induced by Bax expression is abrogated in cells deficient in *UTH1*. These results suggested that Uth1p is involved in mediating the mitophagic effects of Bax [147].

4.3.2. Bax expression leads to vacuolar defects

In addition to changes at the mitochondria, Bax expression in yeast has been reported to induce alterations in vacuolar morphology [139]. This was noted upon analysis of a strain carrying a mutant allele of the *ARL1* gene that is defective in central vacuole formation and membrane trafficking resulting in a delay of programmed cell death with autophagy. This strain was also resistant to cell death induced by Bax expression. Ultrastructural analysis revealed that Bax expression induced autophagic hallmarks that were not observed in the strain mutated at the *ARL1* locus. Also, Bax-induced apoptotic features, including membrane blebbing and chromatin condensation, were more pronounced in strains that were defective in central vacuole formation (i.e. mutated at *ARL1*) [139]. These data suggest that while Bax expression induces both apoptosis and cell death with autophagy, the latter may be a default outcome.

Further evidence for pleiotropic effects of Bax in yeast came from a screen for yeast mutants displaying resistance to the lethal effects of Bax [158]. This resulted in the isolation of 12 Bax-resistant strains that were able to grow on non-fermentative carbon sources, indicating that the phenotype was not due to respiratory defects [158]. Supporting the observations that resistance to Bax is conferred by defects in autophagic and membrane trafficking processes, 8 of the 12 mutants displayed decreased viability in conditions promoting nutrient recycling (i.e. after an incubation period in nitrogen-deprived media). The majority of the Bax-resistant strains also failed to exhibit changes in mitochondrial physiology that are readily observed upon Bax expression in wild type cells (i.e. hyperpolarization and subsequent depletion of $\Delta\Psi_m$). Notably, while most mutants displayed reduced Bax-induced rupture and eventual loss of vacuolar structures, one strain (R6 strain) showed pronounced changes in vacuolar architecture. A set of suppressors of the Bax-resistant phenotype observed in the R6 strain, was found to consist of known components of protein transport stress response machineries. These suppressors promoted Bax-induced disruption of the vacuole, but left the mitochondria unaffected. This indicates that the mechanism by which these suppressors promote Bax sensitivity in the R6 strain is independent of the mitochondria.

A further demonstration that the vacuolar defects associated with Bax expression are independent of apoptosis-inducing effects of Bax on mitochondria comes from a study of the Bax-Inhibiting-Glutathione *S*-transferase (BI-GST) protein, isolated as a high-copy suppressor screen from a tomato cDNA library [78,159]. BI-GST expression confers protection from the effects of Bax, as well as the effects of pro-oxidants in a *YAP1*-dependent manner. Interestingly, while co-expression of Bcl-2 was expectedly sufficient to reverse the mitochondrial defects induced by Bax, the vacuolar alterations persisted. In contrast, Bax-induced aberrant cargo sorting phenotype and vacuolar changes were rescued in cells co-expressing BI-GST. These findings suggest that Bax also has distinctly different functions at the mitochondria and the vacuole.

4.3.3. Bax Inhibitor-1: Further evidence for pleiotropic effects of Bax

Xu and Reed, using a functional lethality screen of a human HepG2 cDNA expression library, identified Bax Inhibitor-1 (BI-1) as the first reported heterologous suppressor of Bax-induced cell death in yeast [59]. This report also demonstrated that BI-1, overexpressed in mammalian cells, is protective from a variety apoptotic stimuli and that its depletion induces apoptosis in HEK293. While human and rat BI-1 had been previously

cloned [61], this was the first demonstration that BI-1 possessed cytoprotective activity. Orthologs of BI-1 have been shown to possess anti-apoptotic functions across the animal, plant and fungal kingdoms [160]. Interestingly, the *Arabidopsis thaliana* BI-1 ortholog (AtBI-1), is capable of suppressing Bax-induced cell death in yeast indicating the ancient evolutionary origins of its anti-apoptotic function [161]. Surprisingly, AtBI-1 has been proposed to act as a dominant negative inhibitor of mammalian BI-1, based on a report of its pro-apoptotic activity upon heterologous expression in human fibrosarcoma HT1098 cells [162]. AtBI-1-induced apoptosis in these cells can be prevented by human BI-1, Bcl-xL, or XIAP co-expression [162]. While this study suggests that the mechanisms of BI-1 function are distinct between yeast and mammalian cells, other plant orthologs of BI-1, such as from tobacco and oilseed rape, are clearly anti-apoptotic in HEK293 cells [163]. Future studies are needed to resolve this discrepancy and would shed further light on the molecular mechanisms of BI-1 function.

Early observations discussed in the study by Xu and Reed also suggested a predominantly ER localization for human BI-1 and were thereafter confirmed in both plant and mammalian cells expressing plant orthologs of BI-1 [163,164]. Interestingly, heterologous expression of AtBI-1 clusters predominantly at the perinuclear region in yeast, suggesting a similar ER localization [164]. Providing strong evidence for the evolutionarily conserved nature of BI-1 function, Ihara-Ohori et al. demonstrated an interaction between AtBI-1 and the ER-resident, Ca^{2+}-handling protein calmodulin in both yeast and plant cells [142]. AtBI-1 mutants unable to interact with calmodulin display no anti-apoptotic activity in yeast, suggesting a requirement for the interaction. Further, this study shows that AtBI-1 serves to regulate stress-induced Ca^{2+} ion fluctuations, and that its anti-apoptotic function in yeast is dependent on *PMR1* and *SPF1*, genes encoding Ca^{2+} pumps which are essential for maintaining ion homeostasis [142]. This is in line with evidence indicating a causal role for ion imbalance in mammalian cell apoptosis [165].

The functional characterization of suppressors of Bax-induced apoptosis can reveal insight into the mechanisms of Bax-mediated apoptosis in yeast. Collectively, the studies of BI-1 function in yeast suggest that Bax-mediated cell death in yeast involves elevations in Ca^{2+} levels, a commonly observed response to many cellular stresses [142]. The subcellular localization of BI-1 is also suggestive of mitochondrial-independent consequences of Bax expression, with effects likely impinging on the endoplasmic reticulum [61]. It is of note that Bax-mediated cyt *c* release is dependent on elevations in ER and mitochondrial Ca^{2+} levels. Therefore, we may speculate that BI-1 acts upstream of Bax in yeast to limit its ability to release cyt *c*, however this remains to be tested. Nevertheless, the BI-1 protein is an exemplary case of the mechanistic insight that can be gained by the continued study of a heterologous suppressor of Bax-mediated apoptosis in yeast.

5. Conclusions and perspectives

Early indications that heterologous metazoan Bcl-2 proteins have similar pro- and anti-apoptotic functions in yeast were largely viewed as artifact. In effect, it has been known for a long time that the expression of numerous heterologous genes in yeast often leads to slow growth and/or cell death likely due to non specific effects believed to be caused by the sheer levels of expression that can be achieved in yeast [166]. Creating further skepticism, yeast lacks endogenous Bcl-2 proteins and was not known to undergo any process resembling apoptosis. The continued study of Bax, coupled to a growing awareness that yeast cells undergo a genetically encoded form of programmed cell death that resembles metazoan apoptosis, has led to an explosion in the study of Bax in yeast. As elaborated here, these studies have increased our understanding of the structure and function of Bax, as well as increased our understanding of the apparent complexity by which Bax functions to kill cells. For example, genetic screens for suppressors of Bax-mediated yeast cell death and endogenous yeast genes that are required for Bax to kill yeast cells has led to the suggestion that processes as diverse as lipid peroxidation, ROS elevation, and ceramide generation are critically involved in the process of yeast apoptosis. The lack of Bcl-2 orthologues in yeast suggests that Bax functions by impinging on components of a conserved apoptotic network. Alternatively, it is tempting to speculate that yeast may contain functional Bax-like proteins, which like the recently recognized yeast metacaspase Yca1p, are not obviously similar to metazoan Bcl-2 proteins. In either case, the study of the Bax in yeast is likely to remain a rich field that will likely give fresh insight into these proteins and yeast apoptosis.

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